Asnaghi, M. A., Duhr, R., Quasnichka, H., Hollander, A., Martin, I., Kafienah, W., & Wendt, D. (2018). Chondrogenic differentiation of human chondrocytes cultured in the absence of ascorbic acid. *Journal of Tissue Engineering and Regenerative Medicine*, *12*(6). https://doi.org/10.1002/term.2671

Chondrogenic differentiation of human chondrocytes cultured in the absence of ascorbic acid

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Short title: Chondrocyte re-differentiation in absence of ascorbic acid

Abstract

Bioreactor systems will likely play a key role in establishing regulatory compliant and cost-effective production systems for manufacturing engineered tissue grafts for clinical applications. However, the automation of bioreactor systems could become considerably more complex and costly due to the requirements for additional storage and liquid handling technologies if unstable supplements are added to the culture medium. Ascorbic acid (AA) is a bioactive supplement that is commonly presumed to be essential for the generation of engineered cartilage tissues. However, AA can be rapidly oxidized and degraded. In this work, we addressed whether human nasal chondrocytes can re-differentiate, undergo chondrogenesis, and generate a cartilaginous extracellular matrix when cultured in the absence of AA. We found that when chondrocytes were cultured in 3D micromass pellets either with or without AA, there were no significant differences in their chondrogenic capacity in terms of gene expression or the amount of glycosaminoglycans. Moreover, 3D pellets cultured without AA contained abundant collagen type II and collagen type I extracellular matrix. While the amounts of collagen II and I were significantly lower (34% and 50% lower) than in pellets cultured with AA, collagen fibers had similar thicknesses and distributions for both groups, as shown by SEM imaging. Despite the reduced amounts of collagen, if engineered cartilage grafts can be generated with sufficient properties that meet defined quality criteria without the use of unstable supplements such as AA, bioreactor automation requirements can be greatly simplified, thereby facilitating the development of more compact, user-friendly, and cost-effective bioreactor-based manufacturing systems.

Keywords: ascorbic acid, collagen, tissue engineering, cartilage, hydroxyproline, nasal chondrocytes

1. Introduction

While engineered tissue grafts hold immense therapeutic potential, a number of obstacles currently exist that inhibit the translation of scientific innovation into commercially viable therapies. Among the obstacles that must be addressed are crucial challenges associated with the manufacturing of engineered grafts (Lysaght *et al.,* 2008, Martin *et al.,* 2014). Bioreactor systems, which automate and standardize bioprocesses, have the potential to overcome key manufacturing challenges and facilitate regulatory compliant and cost-effective production of engineered tissue grafts (Martin *et al.,* 2009, Ratcliffe and Niklason 2002).

The automation of bioreactor systems, however, can become considerably complex due to the complicated liquid handling and storage conditions imposed by unstable compounds used in many culture medium formulations. We have previously found the growth factor TGF-β to maintain high bioactivity even when stored at 37°C for up to three weeks (Vonwil *et al.,* 2008), however, the stability of other medium supplements should also be assessed. Ascorbic acid (AA) is a bioactive supplement typically used in the generation of engineered cartilage, which can be rapidly oxidized to dehydroascorbic acid and further degraded by oxidation or hydrolysis, resulting in short-time stability (Chepda *et al.,* 2001, Fisher and Naughton 2004, Kurano *et al.,* 1990). Several AA derivatives have been developed with protective groups against degradation, such as the more stable but equally bioactive ascorbic acid-2-phosphate (AA2P) (Takamizawa *et al.,* 2004). Despite its increased stability, AA2P is typically added from frozen aliquots at every medium exchange, which drastically complicates bioprocess automation due to the additional need for freezing/thawing modules and the associated liquid handling technology, therefore presenting significant obstacles towards establishing a i) compact, ii) user-friendly, and iii) cost-effective bioreactor-based manufacturing system.

In addition to being a notable antioxidant, AA is thought to induce chondrogenesis via an upregulation of collagen expression, and is therefore added in most protocols for chondrogenic differentiation (Altaf *et al.,* 2006, Cigan *et al.,* 2013, Ibold *et al.,* 2009); an essential process in the production of engineered cartilage grafts. Moreover, AA is involved in the synthesis of collagens, including collagen type II, a key protein in articular hyaline cartilage (Buckwalter and Mankin 1998). In particular, AA serves as a co-factor for the enzymes prolyl hydroxylase and lysyl hydroxlase, which are responsible for the hydroxylation of proline and lysine residues of the collagen propeptide. This hydroxylation is essential for the collagen monomer cross-linking and collagen triple helix stability (Barnes and Kodicek 1972, Englard and Seifter 1986, Kavitha and Thampan 2008).

Nevertheless, it has been shown in a number of model systems that alternative biomolecules are available which could replace AA as an antioxidant and cofactor (e.g., glutathione) (Barnes and Kodicek 1972, Englard and Seifter 1986, Flashman *et al.,* 2010, Martensson *et al.,* 1993, Nytko *et al.,* 2011), and thus AA may not be required for the generation of hyaline cartilage tissues.

In this work, we addressed whether expanded human nasal chondrocytes can re-differentiate, undergo chondrogenesis, and generate a cartilagenous extracellular matrix when cultured *in vitro* in the absence of AA. With an increased understanding of the effects of AA on cartilage tissue engineering, efficient supplementation regimes can be established to define automation requirements, while ensuring graft quality.

2. Materials and Methods

2.1 Cell isolation and expansion

Samples of human cartilage were collected from the nasal septum of 10 patients undergoing rhinoplasty (mean age 37 years, range 21–63 years), after informed consent and in accordance with the local ethical commission (EKBB; Ref.# 78/07). Cartilage biopsies were digested using 0.15% collagenase II (Worthington, UK) for 22 hours at 37°C as previously described (Jakob *et al.,* 2003). After digestion, cells were plated in tissue culture flasks at a density of $1x10^4$ cells/cm^2 and cultured in medium consisting of Dulbecco's Modified Eagle's Medium containing 4.5mg/ml D-glucose and 0.1mM nonessential amino acids (DMEM, Gibco, Life Technologies, Switzerland), 10% fetal bovine serum (FBS), 1mM sodium pyruvate, 100mM HEPES buffer, 100U/ml penicillin, 100µg/ml streptomycin, and 0.29mg/ml L-glutamine (all from Gibco), supplemented with 1ng/ml transforming growth factor beta-1 (TGF-β1) and 5ng/ml fibroblast growth factor-2 (FGF-2) (both from R&D Systems, UK) at 37°C and 5% CO_2 in a humidified incubator (Thermo Scientific Heraeus, USA) (Jakob *et al.,* 2001). When approaching 80% confluence, cells were detached using 0.05% trypsin-EDTA (Gibco), re-suspended in FBS containing 10% dimethyl sulfoxide, transferred to cryovials (Sarstedt, Germany) and stored in liquid nitrogen tanks until further use.

2.2 Chondrogenic re-differentiation

Chondrocytes from frozen aliquots were expanded until passage 2 and re-differentiated by culturing as 3D micromass pellets, which were formed by centrifuging 5E+05 cells at 300x*g* in 1.5ml conical tubes (Sarstedt). 3D micromass pellets were cultured for two weeks either with or without 100µM ascorbic acid 2-phosphate (Sigma) (experimental groups: +AA2P or -AA2P, respectively) in chondrogenic serum-free medium consisting of DMEM containing 1mM sodium pyruvate, 100mM HEPES buffer, 100U/ml penicillin, 100µg/ml streptomycin, 0.29g/ml L-glutamine, 1.25mg/ml human serum albumin (CSL Behring, Switzerland), and 100nM dexamethasone (Sigma, Switzerland), supplemented with 10ng/ml TGF-β1, ITS+1 (10µg/ml insulin, 5.5µg/ml transferrin, 5ng/ml selenium; Gibco), 0.5mg/ml bovine serum

albumin (Sigma), and 4.7µg/ml linoleic acid (Sigma). For an additional experimental group, micromass pellets were cultured with AA2P, and the concentration of AA2P remaining in the culture medium was readjusted to the initial level of 100µM each day (experimental group: Daily AA2P), based on measurements of AA2P (see Section 2.3). As negative controls for chondrogenic re-differentiation, one group of micromass pellets was cultured for two weeks without AA2P and without ITS+1 (experimental group: -AA2P -ITS). For all experimental groups, micromass pellet culture medium was changed twice weekly.

2.3 Ascorbic acid measurements

AA2P was quantified in chondrogenic medium (both in serum-free medium and in 10% FBS containing medium), which was stored at 4°C or 37°C for up to 14 days (0, 1, 2, 4, 7, 10, and 14 days) using the ferric reducing ascorbate assay kit (FRASC; BioVision, Switzerland), according to the manufacturer's instructions with an additional step in order to measure the 2-phosphate form. In addition, AA2P was also quantified in the spent medium of +AA2P micromass pellet cultures at 0, 1, 2, and 3 days. Briefly, to transform AA2P into measurable AA, samples were first incubated with 1unit/100µl of alkaline phosphatase (Sigma, Switzerland) for 10min at 37°C. This step was omitted when measuring the medium concentration of AA without the 2-phosphate group. Ascorbate oxidase or water was then added to generate the AA depleted background or the total oxidant group, respectively. After addition of the ascorbic acid reaction mix, absorbance at 593nm was measured and the concentration of AA2P or AA was calculated from the difference of the total oxidant and the AA depleted group based on an AA standard curve.

2.4 Histology and immunohistochemistry

Micromass pellets were fixed overnight in 4% formalin and embedded in paraffin. Sections 5µm in thickness were stained with Safranin-O for glycosaminoglycans (GAG) and

hematoxylin as a nuclear counterstaining. Immunohistochemistry against collagen type I (No.0863170, MPBiomedicals, France) and collagen type II (No.0863171, MPBiomedicals) was performed using the Vectastain ABC kit (Vector Labs, USA) with haematoxylin counterstaining as previously described (Scotti *et al.,* 2010). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) with an Alexa Fluor 647 dye (Click-iT TUNEL assay; Molecular Probes, Switzerland) was performed on formalin-fixed paraffin-embedded sections, according to the manufacturer's instructions. A positive control with DNA strand breaks was generated by 30min incubation at room temperature with 1 unit of DNase I (Molecular Probes). Immunofluorescence staining against collagen type II was performed on OCT (CellPath, UK) embedded 10µm thick cryosections using the collagen type II primary antibody (MPBiomedicals, France) and a goat anti-mouse Alexa Fluor 546 secondary antibody (Invitrogen), with DAPI as a nuclear counterstain.

2.5 Quantitative RT-PCR

mRNA of chondrocytes was extracted using Quick-RNA Miniprep (Zymo Research, USA), according to the manufacturer's protocol. DNaseI (Zymo Research) was used to remove trace DNA. Isolated RNA was quantified using a NanoDrop spectrophotometer (ThermoFischer Scientific). Reverse transcription into cDNA was done from 3µg of RNA by using 500µg/ml random hexamers (Promega, Switzerland) and 0.5µl of 200UI/ml SuperScript III reverse transcriptase (Invitrogen), in the presence of dNTPs. Real-time PCR was carried out on an ABI Prism 7700 Sequence Detection System (Perkin-Elmer/Applied Biosystems, Switzerland). After initial denaturation at 95°C for 10min, cDNA was amplified for 40 cycles, each consisting of a denaturation step at 95°C for 15sec and an annealing/extension step at 60°C for 60sec. Primers and probes for aggrecan, versican, collagen type I, collagen type II and GAPDH were used with TaqMan Gene Expression Master Mix (Applied Biosystems) as previously described (Martin *et al.,* 2001). Assay on-Demand (Applied Biosystems) was used

to measure the expression of SOX9 (Hs00165814_m1). The threshold cycle (Ct) value of the reference gene GAPDH was subtracted from the Ct value of the gene of interest to derive ΔCt. The relative gene expression of each group normalized to the cells after expansion was calculated as $2^{-\Delta\Delta Ct}$. Each sample was assessed at least in duplicate for each gene of interest.

2.6 Biochemical analyses

Micromass pellets were digested in proteinase K (1mg/ml proteinase K in 50mM Tris with 1mM EDTA, 1mM iodoacetamide, and 10mg/ml pepstatin A) for 16h at 56°C. The sulphated glycosaminoglycan (sGAG) content of pellets was determined by spectrophotometry using dimethylmethylene blue, with chondroitin sulphate as a standard (Barbosa *et al.,* 2003). The DNA content of pellets was measured using the CyQuant cell proliferation assay kit (Invitrogen), with calf thymus DNA as a standard. The amount of sGAG in pellets was then normalized to the DNA content. For hydroxyproline quantification, samples were processed as previously described (Cigan *et al.,* 2013, Hofman *et al.,* 2011). Briefly, proteinase K digested samples were mixed with equal amounts of 12M HCl and hydrolysed at 120°C for 24h. Specimens were then transferred into 96-well plates and left to dry before addition of acetate-citrate buffered chloramine T and incubation for 20min at room temperature. Dimethylaminobenzaldehyde was added and the plates heated for 20min to 60°C. Absorbance was then measured at 550nm. Concentrations were calculated based on a hydroxyproline (Sigma) standard curve.

2.7 Collagen quantification

Pellets were solubilized by an initial digestion in 25µl of 2mg/ml TPCK-treated bovine pancreatic trypsin (in 50mM Tris HCl pH 7.5, 1mM EDTA, 1mM iodoacatamide, 20µg/ml Pepstatin A), for 15h at 37 °C. A further 25µl of freshly prepared trypsin solution was added, and digests were incubated for 2h at 65°C, with intermittent vortexing. Digests were then

boiled for 15min to destroy remaining enzyme activity. The quantity of collagen type II was determined using an inhibition ELISA with a mouse IgG monoclonal antibody to denatured collagen type II (Dickinson *et al.,* 2005). Collagen type I was measured by inhibition ELISA using a rabbit anti-peptide antibody to collagen type I (Hollander *et al.,* 1994).

2.8 Scanning electron microscopy

For scanning electron microscopy (SEM), pellets were fixed for 2 days in 0.1M cacodylate-buffered 2% glutaraldehyde at pH 7.2 at 25°C, then glued onto a Teflon disc with a rapidly curing epoxy glue (Araldite; Huntsman, UK). Next, the specimens were placed in a cryostat microtome to trim off approximately 150µm of the outermost cartilage layer parallel to the support surface to assess the central region of the samples. Proteoglycans were then extracted in 100mM Soerensen's phosphate buffer (pH 7.2) containing 1mg/ml bovine hyaluronidase (type I, Sigma), 1mg/ml trypsin (type I, Sigma) and 0.01% NaN_3 at 37°C for 3 days. After dehydration in graded ethanol series and critical point drying, samples were sputter-coated with 3–5 nm platinum and examined by SEM (Hitachi S-4800 FEG, Japan), operated at 1.5–5kV accelerating voltage in immersion mode (Stolz et al., 2009, Strobel et al., 2010). The collagen fibers within micromass pellets were assessed qualitatively based on the acquired SEM images.

2.9 Statistical analysis

Data are presented as mean and standard deviation of independent experiments with cells from at least 2 different donors. For each analysis at least 3 replicate micromass pellets were used per condition. Statistical analysis was performed using RStudio version 1.1.149 (RStudio, Boston, MA; http://www.rstudio.com). The effects of the ascorbic acid treatment groups were assessed using a linear mixed effect model. Treatment group was defined as the fixed effect and cell donor defined as the random effect. Measurement values were log10

transformed and then fit with the mixed effect model using the R package lme4. Differences were assessed using a one-way ANOVA followed by a post-hoc Tukey test using the R package multcomp. Differences between groups were considered statistically significant for $p<0.05$.

3. Results

3.1 AA2P concentration in culture media

In order to test the availability of AA in chondrogenic medium formulations, AA stability was determined in stored medium and spent pellet culture medium, with or without FBS, for the two week culture period required for automated bioreactor-engineered cartilage (Tonnarelli *et al.,* 2016). When media was supplemented with AA, which lacks the protective 2-phosphate group, levels were below the detection limit in both stored and spent pellet culture medium at all time points, indicating a rapid degradation and/or consumption of this medium supplement (data not shown). For the remainder of the study, we therefore exclusively tested media supplemented with AA2P, and not AA. In medium containing serum, AA2P was rapidly degraded. AA2P was particularly unstable when stored in medium containing serum at 37°C, dropping to less than 10% of the initial concentration within 24 hours (Fig. 1a). In serum-free culture medium, the concentration of AA2P remained constant for 2 weeks of storage, both at 4°C and 37°C. However, when culturing micromass pellets in serum-free culture medium, the concentration of AA2P decreased by almost 50% within the first 24 hours of culture, and further declined to less than 25% of the initial concentration by day 3 (Fig. 1b). Since a constant concentration of AA or AA2P was not maintained in the presence of cultured chondrocytes without repeated supplementation (which would ultimately impose critical manufacturing challenges when using a closed bioreactor system), we investigated whether the continual presence of AA2P was necessary to maintain a chondrogenic phenotype and for

the production of components required for engineered cartilage. This question was addressed by using nasal chondrocyte pellet cultures in serum-free medium.

3.2 Cell viability

Since AA is a potent antioxidant that prevents oxidative stress in cells, the effect of its absence on cell viability was tested in micromass pellet cultures under normoxic conditions. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was similar in pellets cultured with (+AA2P and Daily AA2P) or without AA2P (-AA2P), with only a few instances of DNA fragmentation detected among all conditions with ITS (Fig. 2a); whereas in the absence of both AA2P and ITS (-AA2P -ITS) all cells exhibited oxidative stress. In addition, the DNA content of all pellets cultured with ITS was the same irrespective of AA2P supplementation, indicating that cell number was unaffected (Fig. 2b).

3.3 Gene expression and glycosaminoglycan deposition

No significant differences were found in the expression of genes associated with either chondrogenic differentiation (collagen type II, aggrecan and sox-9) or chondrocyte dedifferentiation (collagen type I and versican) irrespective of AA2P supplementation (Fig. 3a). Safranin-O staining of micromass pellets showed similar cell densities and uniform GAG deposition irrespective of the AA2P supplementation (Fig. 3b). Negative control -AA2P -ITS pellets were filled with extracellular matrix that was negative for GAG staining. Biochemical quantification of pellets showed that the sGAG/DNA content was consistent with histological assessment and unaffected by AA2P supplementation. In contrast, -AA2P -ITS pellets had significantly lower sGAG/DNA (Fig. 3c).

3.4 Collagen extracellular matrix

Since ascorbic acid is a cofactor in the hydroxylation of proline during collagen synthesis, we tested the effects of AA2P on collagen deposition and hydroxylation. Immunohistochemical analysis showed that micromass pellets stained positive for both collagen type II and collagen type I, with intensities and staining patterns unaffected by AA2P supplementation (Fig. 4). Collagen type I was distributed mainly on the edge of the pellets, while collagen type II was abundantly present throughout the whole pellet, except for a thin outer rim, indicating deposition of a cartilage like matrix. Removal of ITS resulted in no positive collagen type II or I staining.

Quantification of pellet collagen type II and collagen type I by specific immunoassays confirmed deposition of both proteins even in the absence of AA2P. The amount of collagen type II and collagen type I in -AA2P pellets were 34% less ($p<0.01$) and 50% less ($p<0.001$) than in +AA2P pellets, respectively (Fig 5a and 5b). The amount of collagens in the Daily AA2P pellet group were similar and not significantly different from the +AA2P group. In contrast, -AA2P -ITS pellets contained significantly less collagen type II and virtually no collagen type I. Post-translational hydroxylation of collagens was confirmed by the quantification of the hydroxyproline/collagen content in pellets, which was similar for all experimental conditions, irrespective of the AA2P condition (Fig. 5c). In contrast, the hydroxyproline/collagen content was significantly lower in negative control pellets (-AA2P -ITS).

3.5 Collagen secretion

Since removal of AA2P could affect deposition of collagen within the extracellular matrix due to impaired collagen secretion, we used high magnification immunofluorescence to assess intracellular and extracellular collagen type II accumulation. As seen in Figure 6, collagen type II was evenly distributed in the extracellular matrix, but no intracellular retention of

vesicle-like structures staining positive for collagen type II could be observed, independent of the AA2P condition. No extracellular or intracellular collagen type II was detected in the negative control pellets (-AA2P -ITS).

3.6 Collagen fibril network

We investigated whether the absence of AA2P had an effect on the collagen fibrils deposited within the micromass pellets. As seen in Figure 6, SEM imaging of micromass pellets showed dense networks of collagen fibrils, which appeared to have similar thicknesses and distributions for all AA2P conditions. In contrast, no collagen fibrils could be observed in the negative control pellets (-AA2P -ITS).

4. Discussion

In this work, we have shown that AA2P was rapidly degraded in medium used for culture of engineered cartilage tissues. However, when chondrocytes were cultured in 3D micromass pellets either with or without AA2P, we found no significant differences in their chondrogenic capacity in terms of gene expression and sGAG deposition. Interestingly, pellets cultured without AA2P contained abundant collagen type II and collagen type I extracellular matrix. While the amount of these collagens was significantly lower in -AA2P pellets than those cultured with AA2P, the network of collagen fibers appeared quite similar.

AA is known to be an unstable component of cell culture medium due to a fast degradation process catalyzed by iron and copper ions (Fisher and Naughton 2004). AA2P, with the protective phosphate group, has been shown to be more stable (Takamizawa *et al.,* 2004), however the stability can be affected by specific components of the culture medium. We have demonstrated that AA2P was stable in serum-free medium for up to two weeks when stored at either 4°C or 37°C. That stability was drastically reduced in medium containing serum, likely

due to the presence of phosphatases in the serum (Chepda *et al.,* 2001). When culturing chondrocytes in micromass pellets, the AA2P concentration was reduced by half after 24 hours, even in serum-free medium. It is likely that membrane-bound or secreted phosphatases degraded AA2P to AA, which was then either rapidly degraded or taken up by the cells (Takamizawa *et al.,* 2004). To account for the depletion of AA2P during culture, in subsequent experiments we included an experimental group in which the level of AA2P was measured and adjusted daily to the initial concentration (i.e., 100μM) in order to assess the effects of a more constant level of AA2P in the culture medium. While the number of cells would likely have an impact on the level of AA2P depletion throughout culture, the ratio of cell number to culture medium volume (1E+06 cells/ml) used in this study has clinical relevance, since it is the same ratio used in the manufacturing of cartilage grafts for clinical studies (Mumme *et al.,* 2016).

Following two weeks of micromass pellet culture under normoxia, we observed no differences in apoptosis, necrosis, or DNA content, regardless of the AA2P supplementation regime. Endogenous antioxidants such as glutathione have likely compensated for the absence of AA2P and provided sufficient protection against reactive oxygen and nitrogen species (Espinosa-Diez *et al.,* 2015).

We compared de-differentiated monolayer expanded chondrocytes with re-differentiated chondrocytes in micromass pellets and found an upregulation of genes associated with chondrogenic differentiation (collagen type II and aggrecan) in pellets. Interestingly, the expression of all genes assessed, whether chondrogenic or not, was similar for pellets cultured with or without AA2P. Biochemical and histological assessments also indicated no differences between +AA2P, Daily AA2P, or -AA2P pellets. In the presence of ITS, with or without AA2P, chondrocytes were rounded and embedded within lacunae, and surrounded by

dense GAG matrix; indicating that the chondrogenic capacity of human nasal chondrocytes was not significantly affected by AA2P.

Immunohistochemical and ELISA assays confirmed that chondrocytes could produce collagen type II and collagen type I proteins in the absence of AA2P, although to lesser extent than when cultured with AA2P. Quantification of collagens type II and I by ELISA enabled collagen to be determined independent of hydroxyproline quantification (Dickinson *et al.,* 2005, Hollander *et al.,* 1994), thus enabling the proportion of hydroxyproline in collagens to be calculated. This calculation was based on the assumption that the majority of collagen in pellets was type II and I, however it is possible that other collagens (e.g., type III and XII) were also present in small amounts (Taylor *et al.,* 2015). Additional biochemical analysis indicated that the amount of hydroxyproline/collagen was similar for +AA2P, Daily AA2P, -AA2P, suggesting that the enzyme proline hydroxylase was functional even in the absence of AA2P. These findings are consistent with previous studies demonstrating that AA was not essential as a co-factor for prolyl hydroxylase under certain conditions. In fact, mutant mice lacking the final enzyme for AA synthesis were shown to have normal collagen production, and the absence of AA had no direct effect on collagen hydroxylation in guinea pigs (Dickinson *et al.,* 2005, Parsons *et al.,* 2006, Peterkofsky 1991). Other molecules present in the medium such as glutathione or cysteine may have compensated for the lack of a co-factor, even if at a lower activity (Barnes and Kodicek 1972). Indeed, lysyl hydroxylase can function in the absence of AA for a short period (Puistola *et al.,* 1980). Alternatively AA could be recycled over long durations, which could also explain why collagen production without ascorbic acid supplementation has been observed *in vivo* (McNulty *et al.,* 2005, Qutob *et al.,* 1998). Interestingly, no increase in collagen deposition (Fig. 4) or hydroxylation/collagen ratio (Fig. 5) was observed when maintaining the concentration of AA2P on a daily basis close to initial levels (Daily AA2P); despite the observed reduction in

available AA after 24 h of pellet culture (Fig 1b). This indicates that the applied regime of twice weekly medium exchanges, was sufficient to reach saturation of AA-dependent mechanisms, before available AA was completely diminished.

Despite similar gene expression, reduced amounts of collagen type II and collagen type I in -AA2P pellets could have resulted from inhibited collagen secretion (Bentovim *et al.,* 2012). Based on immunofluorescence staining for collagen type II, no intracellular retention of vessel-like structures could be detected (Gelse *et al.,* 2008), suggesting collagen secretion may not have been significantly impaired in the absence of AA2P. However, additional analyses should be performed in order to more closely investigate potential impaired collagen secretion, such as TEM imaging or an investigation of the unfolded protein response (UPR).

The unique biomechanical features of articular cartilage largely depend on its highly organized molecular and structural composition (Buckwalter and Mankin 1998). Based on SEM imaging, all micromass pellets qualitatively appeared to have similar fiber thicknesses and distributions, regardless of AA2P supplementation. Together with the similar hydroxylation profiles, this suggests that the extracellular matrix engineered with or without AA2P could potentially have similar biomechanical properties (Berg and Prockop 1973). This hypothesis remains to be investigated in future studies. Furthermore, the analysis of collagen crosslinks would provide insight into the integrity of collagen fibrils in the absence of AA2P, since crosslink formation requires hydroxylation of lysine residues, and hydroxylysine derived immature crosslinks such as lysinonorleucine provide tensile strength to collagen fibrils (Davison 1989). Maturation of collagen crosslinks into tri and tetravalent hydroxyl-pyridinoline crosslinks increases collagen fibril strength further (Reiser *et al.,* 1987), but this condensation reaction is unlikely to occur within two weeks of collagen synthesis (Murdoch *et al.,* 2016).

In this work, we have shown that de-differentiated human nasal chondrocytes cultured as micromass pellets without AA2P, could re-differentiate and generate cartilaginous tissue. The engineered tissues had similar properties to those generated in the presence of AA2P, with the exception of lower collagen content. Although the amount of collagen type II was reduced by 34% in the absence of ascorbic acid, the question remains whether this slight reduction in collagen content would have any clinical impact. Clinical studies aimed at understanding the effect of the maturation stage of engineered cartilage tissues on cartilage repair will help to address the lingering question in cartilage tissue engineering "how good is good enough?". If engineered cartilage grafts can be generated with sufficient properties to meet defined quality criteria without the use of unstable medium components such as ascorbic acid, bioreactor automation requirements can be greatly simplified, thereby facilitating the development of a more compact, user-friendly, and cost-effective bioreactor-based manufacturing system.

Acknowledgements

This project has received funding from the European Union's Seventh Program for research, technological development, and demonstration under grant agreement No. 278807 (BIO-COMET) as well as the Fonds National de la Recherche Luxembourg (4090751). The authors would like to acknowledge Evi Bieler from the Centre of Microscopy at the University of Basel for assistance with electron microscopy, and Dr. Martin Haug from the Department of Surgery at the University Hospital Basel for providing the nasal cartilage biopsies.

Figure Legends

Figure 1. Availability of AA2P in stored medium and in micromass pellet cultures. (a) Concentration of AA2P in serum-free medium (SF) or 10% fetal bovine serum-containing medium (CM) stored at 4°C or 37°C for up to 14 days. (b) Concentration of AA2P in spent serum-free medium of micromass pellet cultures.

Figure 2. (a) TUNEL staining (red) and DAPI nuclear counterstaining (blue) of micromass pellets after 2 weeks of chondrogenic culture. Arrows point to TUNEL positive staining. (Scale bar 50μm). (b) DNA quantification of micromass pellets. Data are presented as mean ± standard deviation.

Figure 3. Gene expression and glycosaminoglycan content following two weeks of micromass pellet culture. (a) The expression of genes associated with differentiated (collagen type II, sox-9, aggrecan) and de-differentiated chondrocytes (collagen type I, versican) after 2 weeks of chondrogenic differentiation. (b) Safranin-O staining of histological sections of micromass pellets. (Scale bar indicates 200μm.) (c) sGAG/DNA of micromass pellets normalized to +AA2P values of the same donor. Data are presented as mean ± standard deviation. (*indicates statistically significant differences from other groups with $p<0.05$)

Figure 4. Collagen type II and collagen type I immunohistochemistry of micromass pellets after two weeks of culture. (Scale bar indicates 200μm.)

Figure 5. Quantification of (a) collagen type II, (b) collagen type I, and (c) the ratio of hydroxyproline to collagen, following two weeks of micromass pellet culture. Values were normalized to the respective value of +AA2P of the same donor. Data are presented as mean ± standard deviation. (*indicates statistically significant difference from +AA2P with $p<0.05$)

Figure 6. (a) Cryosections of micromass pellets stained by immunofluorescence against collagen type II (red) and DAPI nuclear counterstain (blue). (Scale bar indicates 10μm.) (b) Scanning electron microscopy of micromass pellets showing the network of collagen fibrils. (Scale bar indicates 300nm)

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Figure 1

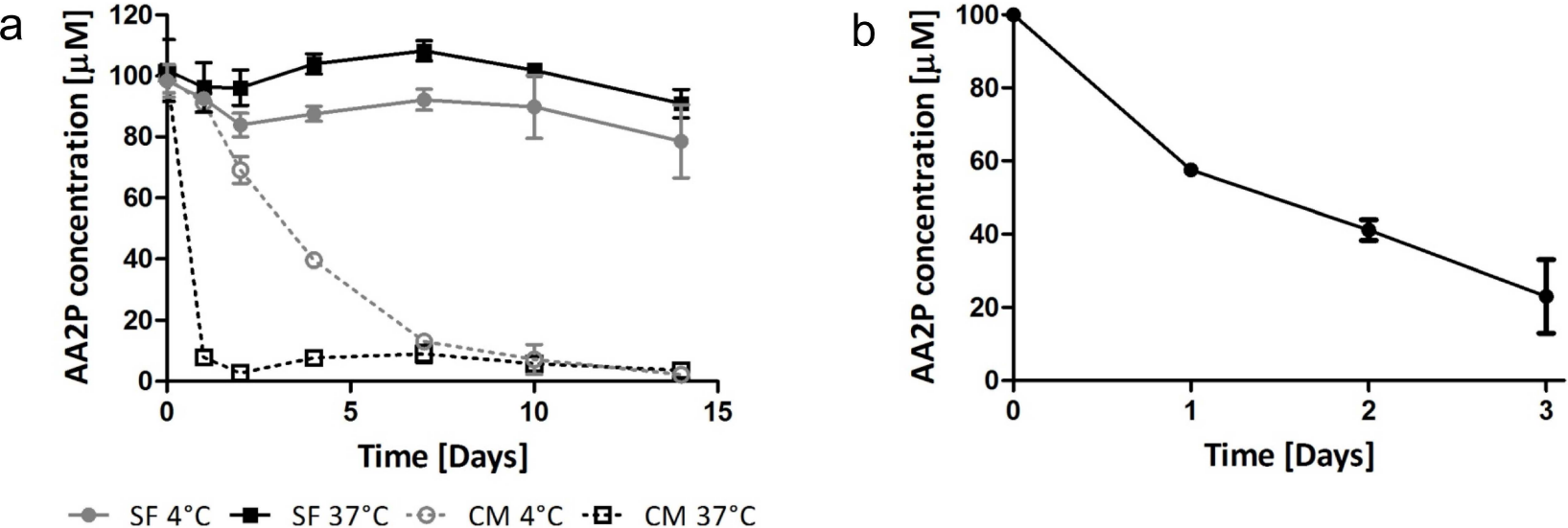
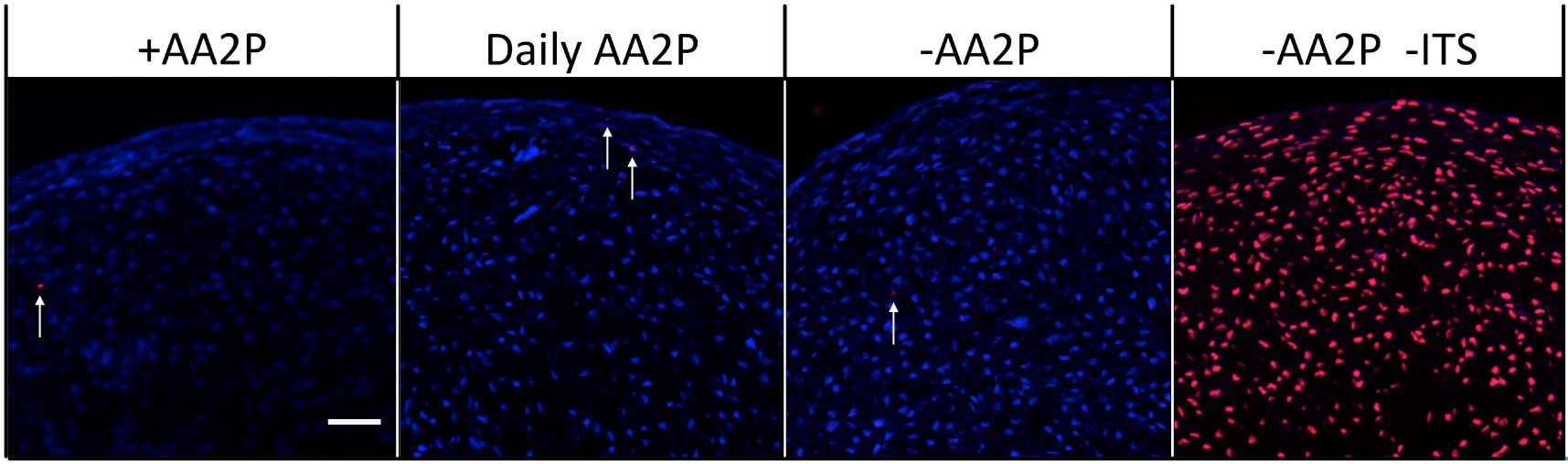

a
AA2P concentration [µM]
120
100
80
60
40
20
0
0
5
10
15
Time [Days]
SF 4°C
SF 37°C
CM 4°C
CM 37°C
b
AA2P concentration [µM]
100
80
60
40
20
0
0
1
2
3
Time [Days]

Figure 2

a


+AA2P
Daily AA2P
-AA2P
-AA2P -ITS

b

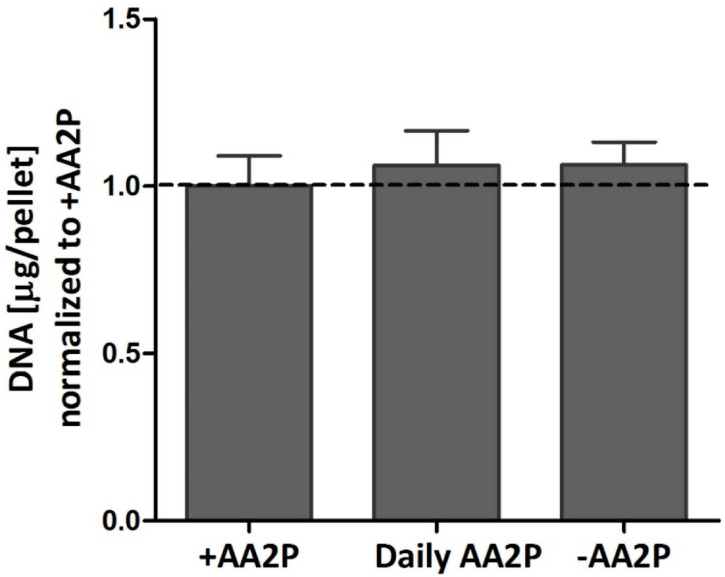

DNA [µg/pellet]
normalized to +AA2P
1.5
1.0
0.5
0.0
+AA2P
Daily AA2P
-AA2P

Figure 3

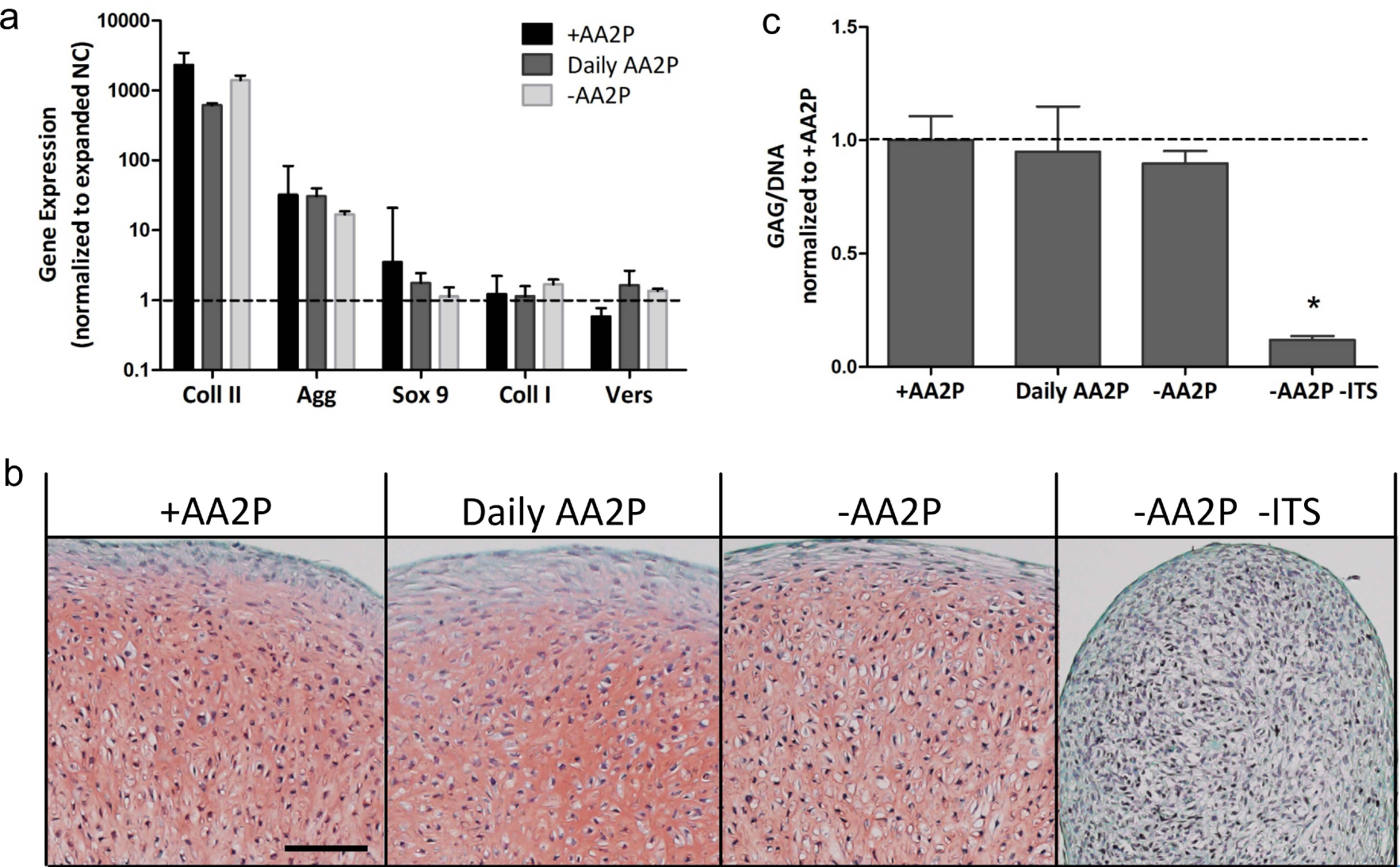

a
Gene Expression
(normalized to expanded NC)
10000
1000
100
10
1
0.1
+AA2P
Daily AA2P
-AA2P
Coll II
Agg
Sox 9
Coll I
Vers
c
GAG/DNA
normalized to +AA2P
1.5
1.0
0.5
0.0
*
+AA2P
Daily AA2P
-AA2P
-AA2P -ITS
b
+AA2P
Daily AA2P
-AA2P
-AA2P -ITS

Figure 4

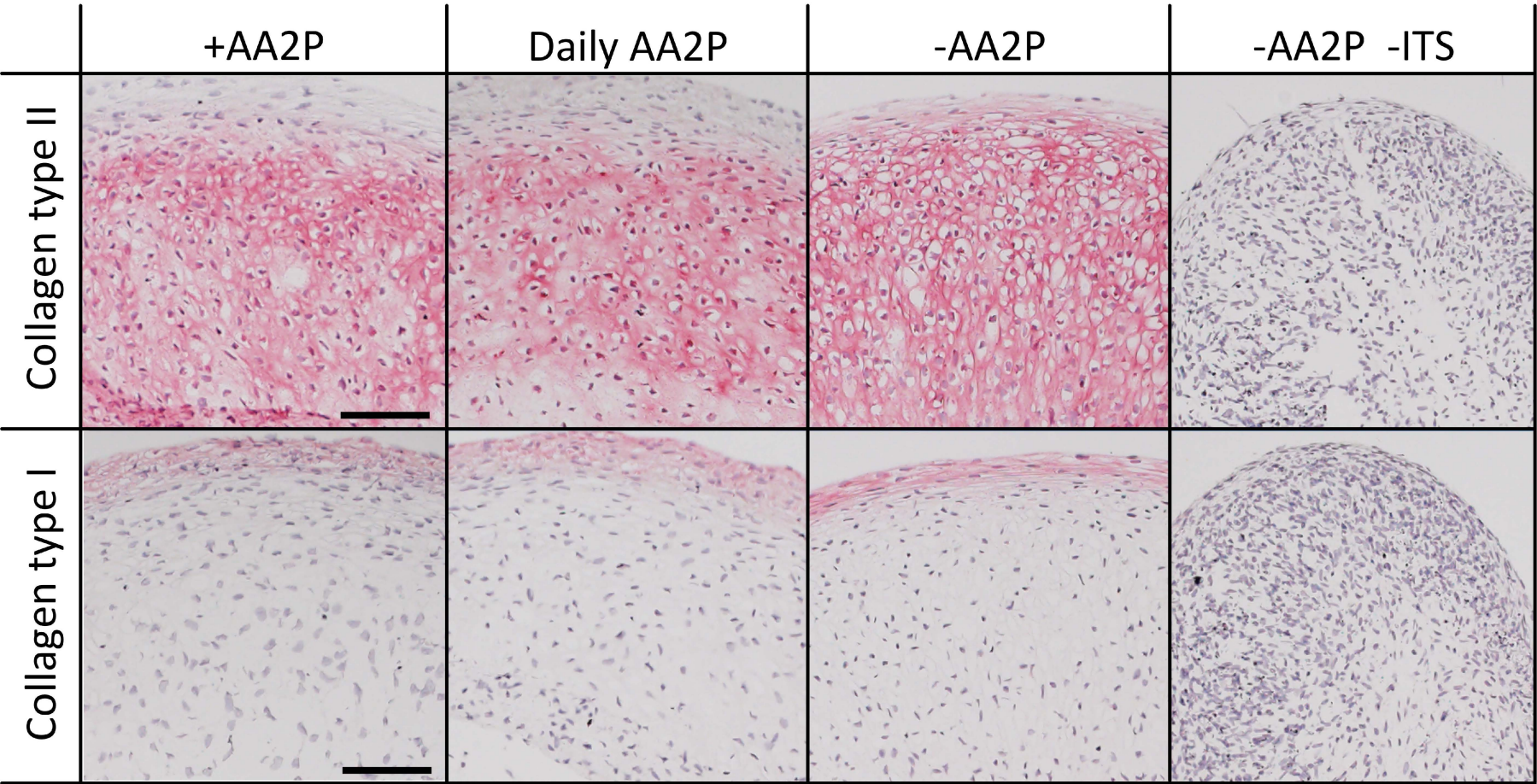

+AA2P
Daily AA2P
-AA2P
-AA2P -ITS
Collagen type II
Collagen type I

Figure 5

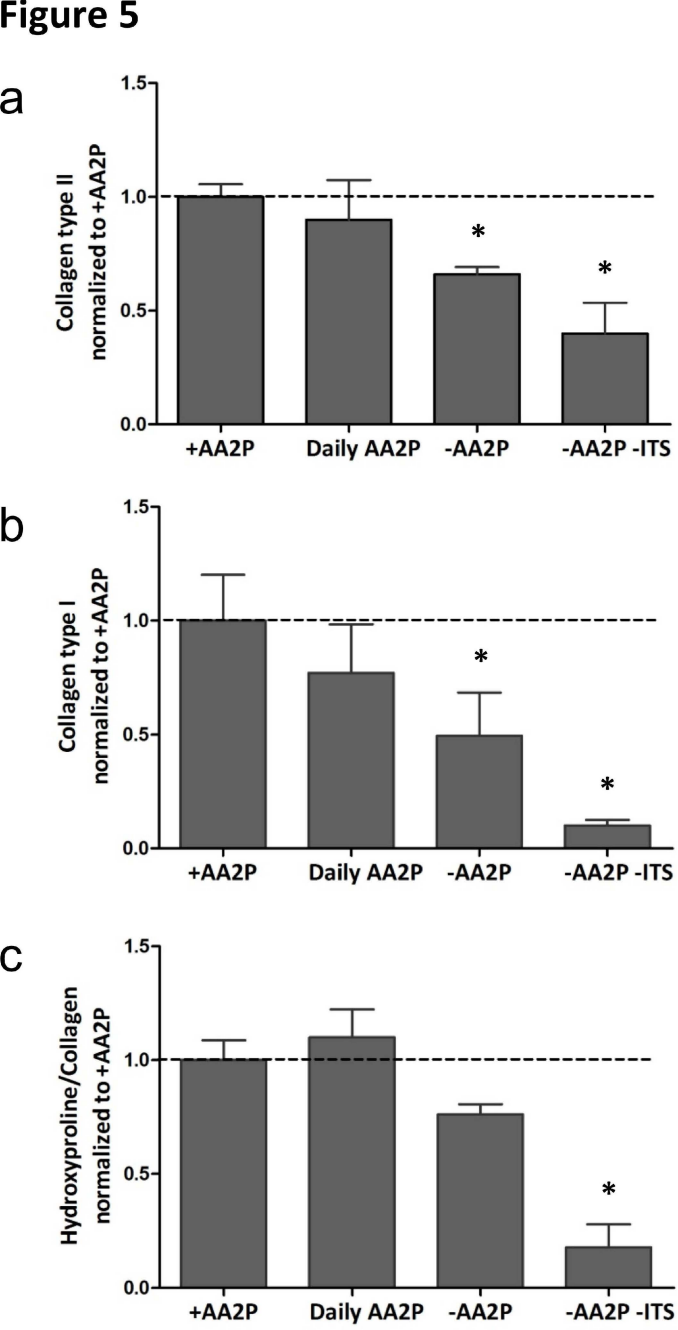

a
Collagen type II normalized to +AA2P
1.5
1.0
0.5
0.0
*
*
+AA2P
Daily AA2P
-AA2P
-AA2P -ITS
b
Collagen type I normalized to +AA2P
1.5
1.0
0.5
0.0
*
*
+AA2P
Daily AA2P
-AA2P
-AA2P -ITS
c
Hydroxyproline/Collagen normalized to +AA2P
1.5
1.0
0.5
0.0
*
+AA2P
Daily AA2P
-AA2P
-AA2P -ITS

Figure 6